Monoclonal antibody and assay for detecting PIIINP

State of the art

The N-terminal procollagen (III) propeptide (PIIINP) molecule is a proteolytic fragment emanating from the specific cleavage of procollagen (III) by N-proteinase after exocytosis. The present invention relates to antibodies binding to this N-terminal end and to an assay using these antibodies

Collagens I and III are synthesized as prepropeptides and are extensively modified posttranslationally. Among the specific intracellular modifications are glycosylations, enzymatic hydroxylation reactions involving lysine and proline in its 3- and 4-positions, and the specific proteolytic removal of the leader peptide. The modified propeptides spontaneously assemble into $[\alpha_1(III)]_3$ homotrimers in the case of collagen (III), and mostly $[\alpha_1(I)]_2\alpha_2(I)$ heterotrimers as well as - to a lesser extent - $[\alpha_1(I)]_3$ homodimers in the case of collagen (I) in the microsomal compartments. After exocytosis, the propeptide is first cleaved at the C-terminus of the nascent collagen and then at the N-terminus by a set of specific endoproteases. Remarkably, procollagens I and II are cleaved by a proteinase activity distinct from the N-proteinase activity specific for procollagen (III) (Peltonen *et al.*, 1985). A candidate gene for a human collagen (III)-specific N-proteinase has recently been suggested (Scott *et al.*, 1996). A putative cDNA sequence for bovine procollagen (I) N-proteinase is available (Colige *et al.*, 1996).

PIIINP occurs as a trimer consisting of three identical monomeric PIIINP subunits that are linked by intermolecular disulfide bridges. PIIINP in turn is structurally divided into three domains. The most N-terminally located domain (Col1) consists of a globular structure linked by several intramolecular cystine bridges. Col3 is the intermediate domain and possesses a collagen-like structure characterized by periodic Gly and Pro residues. This domain assembles into a characteristic triple-helical collagen-like structure characterizing the Col3 domain. The Col2 domain encompasses the parts of the procollagen telopeptide region proximal to the N-

proteinase cleavage site. The three monomeric PIIINP subunits are assembled as parallel peptide strands in this domain. Characteristically, the Col2 domain contains two cystein residues that are both involved in intermolecular disulfide bridge formation and that are solely responsible for the trimeric structure of PIIINP (Kühn *et al.*, 1982).

The entire cDNA sequence encoding PIIINP was PCR-cloned from a human aorta cDNA library (QUICK-Clone cDNA, Clontech, USA), sequence-verified and subcloned into the bacterial phagemid vector pBluescript SK$^-$ (Stratagene, USA). Sequence information has been obtained from the DNASTAR program (Genbank accession # X14420; Ala-Kokko *et al.*, 1989, figure 1).

State of the art: measurements of collagen fragments

Collagen (III) is the characteristic collagen of parenchymal organs and is the second most prevalent collagen in fibrotic tissues. Although collagen (I) is the most prevalent scar-forming collagen and although collagen (I) is upregulated even more drastically in liver fibrosis than collagen (III) it occurs in large amounts in bone (Uitto *et al.*, 1986) and is therefore of limited value in the differential diagnosis of fibrotic processes in parenchymal organs. C-terminal procollagen (I) propeptide serum determinations have therefore been used primarily for the monitoring of disorders of bone metabolism (Eriksen *et al.*, 1993).

Serum levels of the circulating N-terminal procollagen (III) propeptide (PIIINP) are already established as a serum parameter in liver fibrosis patients to estimate the amount of collagen deposition in this organ (e.g. Plebani and Burlina, 1991).

Elevated circulating PIIINP levels may, however, also originate from the cleavage of deposited collagen (III) and thus reflect fibrolysis rather than fibrogenesis (Schuppan, 1991). This is due to the fact that PIIINP is present on the surface of collagen (III) after its deposition in the extracellular matrix (Fleischmajer *et al.*, 1986). The molecular masses of the PIIINP species emanating from fibrolysis seem to be distinct

from the species generated in fibrogenesis and include higher as well as lower molecular mass species that circulate in the plasma (Niemelä *et al.*, 1982). Although PIIINP serum determinations are fairly well established for the monitoring of liver fibrosis in various underlying diseases such as primary biliary cirrhosis (e.g. Davis and Madri, 1987), chronic hepatitis B and C (Murawaki *et al.*, 1995, Jeffers *et al.*, 1996), and alcoholic liver disease (Savolainen *et al.*, 1984), they are of little help in the establishment of the diagnosis. This is due to two different characteristics of PIIINP serum determinations: 1. circulating PIIINP levels seem to correlate with the acute phase of liver inflammation when excessive collagen deposition is not yet visible histologically and may in fact never become manifest (Savolainen *et al.*, 1984, Hansen *et al.*, 1995) 2. increased circulating PIIINP levels in clinical settings can indicate fibrolysis rather than fibrogenesis because uncleaved PIIINP is present on the surface of collagen (III) fibers and is liberated in the process of collagen degradation (Davis and Madri, 1987).

The diagnostic value of PIIINP determinations has been debated extensively. From molecular sieve experiments with patient sera it has been shown that serum assays recognizing PIIINP detect three different molecular weight forms. The fraction containing lower molecular weight species consists of monomeric Col1 domains. The absolute amount of this circulating Col1 domain is relatively constant in healthy volunteers as well as in patients with chronic active hepatitis and acute alcoholic hepatitis. In addition to the circulating Col1 domain fragment, the antibodies also recognize higher than trimeric PIIINP species in the sera. The exact molecular nature of these high molecular weight species is not known. The relative proportion of the high molecular weight species appears to vary depending on the type of liver disease. Trimeric PIIINP emanating from collagen synthesis and cleaved by N-protease is usually the most abundant PIIINP species but its relative proportion is not constant (Niemelä *et al.*, 1982).

An additional problem with the undifferentiated PIIINP serum levels is the unsettled question if PIIINP is correlated with ongoing collagen (III) neosynthesis or whether it is better correlated with manifest collagen deposition in the liver. While some

investigators have published that PIIINP levels are best correlated with collagen (III) mRNA (Hayasaka *et al.*, 1991) other studies do not support these observations.

A number of patents have dealt with the problem of PIIINP determinations from patient sera and with methods to improve the diagnostic validity of these determinations. For the measurement of PIIINP in sera of patients with liver diseases several different PIIINP radioimmunoassays have been reported. EP 0004940A1 by Timpl *et al.*, 1979, describes a non-equilibrium inhibition radioimmunoassay based on a bovine antigen-antibody system which shows cross-reactivity with human PIIINP. The procedure disclosed in this patent was subsequently published by Rohde *et al.*, 1979, and was used to develop the RIAgnost PIIIP assay of Behring AG, Marburg, Germany. However, the method does not allow a precise determination of trimeric PIIINP in patient sera since the polyclonal antibodies used in the assay recognize both intact PIIINP and degradation products of PIIINP, in particular the monomeric Coll domain. In addition, the avidity of the antiserum for the monomeric degradation product is lower than for intact PIIINP. Thus, serum samples show a less steep inhibition curve when compared with the PIIINP standard and the PIIINP concentration has to be estimated by the 50% intercept method which requires three serum dilutions.

In order to overcome the problem with the flat inhibition curve of serum samples an assay variant using antibody Fab-fragments has been developed. The method disclosed in EP 0089008A2 by Timpl *et al.*, 1983, and subsequently published by Rohde *et al.*, 1983, takes advantage of an antiserum that has been generated by immunization of mice with Coll. The antiserum is used to generate antibody Fab-fragments that have an almost equal avidity for Coll and for intact PIIINP. Therefore, parallel inhibition curves are obtained for serum samples and standards and only a single dilution is required. However, the diagnostic value of this assay is inferior to the RIAgnost PIIIP assay because the Fab-assay does not efficiently differentiate between active and inactive liver diseases.

The low diagnostic value of the Fab-assay has led to the assumption that instead of using an antiserum with equal affinity to PIIINP and Coll, it might be better to use an

antiserum which does not recognize Col1 at all. The European patent application EP 0298210A2 by Brocks and Timpl, 1988, describes a method how to raise an antiserum that recognizes intact PIIINP and some undefined procollagen type III species with a higher molecular weight than PIIINP, but not Col1. The antiserum is obtained after immunization of mice with a peptide of defined sequence. However, the peptide claimed is of rat or bovine origin and the antiserum obtained after immunization does not show sufficient crossreactivity with human PIIINP. Therefore, the assay is not applicable to human samples (Brocks *et al.*, 1993).

Similarly, in European patent application EP 0304292A2 by Risteli and Risteli, 1988, a method is claimed that allows the generation of polyclonal antibodies that have almost no affinity for the PIIINP degradation product Col1. In particular it is claimed that the desired antibodies are obtained after immunization of mice with a trimeric aminoterminal propeptide free from proteolytic enzymes. Moreover, EP 0304292A2 describes an equilibrium RIA which is easier to perform than the non-equilibrium RIAs mentioned in the previous patent applications. A detailed description of the assay, which is commercially available by Orion Diagnostica, Espoo, Finland, was published by Risteli *et al.*, 1988.

The production of two monoclonal antibodies as described in EP 0289930A2 by Brocks *et al.*, 1988, and EP 0339443A2 by Brocks *et al.*, 1989, has allowed the development of an immuno radiometric assay. The reaction pattern of both antibodies against PIIINP separated by gel chromatography is very similar and characterized by the absence of reactivity against Col1. The assay is commercially available as the coated tube RIAgnost PIIIP assay from Behring Diagnostica, Marburg, Germany. The authors also claim the combination of these two antibodies and other unspecified antibodies against PIIINP in sandwich immuno assays.

State of the art: recombinant production of PIIINP

All antibodies described in the literature and in various patents have been raised against PIIINP purified from human or bovine sources. Therefore, the binding epitope of the antibodies is not well characterized.

A number of publications have reported the recombinant production of procollagens. One publication (Lee *et al.*, 1990) claims the production of a collagen α_2(I) mutant in the C-proteinase cleavage site in A2 cells derived from the rat liver epithelial cell line W8 which is deficient for collagen α_2(I). Two recent publications report the recombinant expression of collagen α_1(III) minigenes (Lees and Bulleid, 1994; Bulleid *et al.*, 1996). The sole recombinant expression of the N-terminal procollagen α_1(III) propeptide, e.g. for the purposes of selective immunizations, or the expression of truncated PIIINP, e.g. for the purpose of epitope-mapping, has not been reported. Immunizations have only been carried out with arbitrarily chosen synthetic peptides of uninvestigated secondary structure.

Although immunizations were in our case performed with an oligopeptide that presumably occured as a monomeric molecule, the resulting antibodies may still preferentially recognize trimeric PIIINP. This may be due to the fact that one antibody can simultaneously bind to two identical epitopes on adjacent chains (Rohde *et al.*, 1983).

A synthetic peptide from the Col1 region (N-ICESCPTGGQNYSP-C) has been used to raise antibodies that are claimed to be directed against intact PIIINP and that appear not to crossreact with monomeric PIIINP forms (EP 0298210A2 by Brocks and Timpl, 1988). Later, the same authors have published that the antibodies directed against this peptide do not recognize human PIIINP (Brocks *et al.*, 1993).

From the literature cited above it becomes clear that systematic epitope mapping approaches utilizing recombinant DNA technology have not been reported for PIIINP before.

State of the art: antibodies against PIIINP

There is considerable interest in antibodies generated against PIIINP. One source (Rohde *et al.*, 1979) reports the generation of polyclonal antibodies reactive against PIIINP and claims its use as a diagnostic tool for PIIINP determinations in human serum samples. For immunizations, the Coll domain purified from bovine type III procollagen was used. The results of this investigation were also the subject of EP 000490A1 by Timpl, 1979.

The generation of antibodies against the synthetic oligopeptide fragment (N-ICESCPTGGQNYSP-C) has been the subject of EP 0298210A2 by Brocks and Timpl, 1988. Antibodies recognizing this antigen were claimed to recognize intact PIIINP in rodent sera as well. However, the antibodies were not crossreactive with human PIIINP (Brocks *et al.*, 1993).

EP 0289930 by Brocks *et al.*, 1988, and EP 0339443 by Brocks *et al.*, 1989, claim the generation of two monoclonal antibodies that are selective for the recognition of intact human PIIINP in various body fluids. In particular, they claim the generation of monoclonal antibodies that are directed against an epitope that is not located on Coll in EP 0289930 and in EP 0339443. While the antibody claimed in EP 0289930 exclusively recognizes higher molecular weight species and intact PIIINP while not reacting with Coll degradation products, the antibody claimed in EP 0339443 recognizes an additional PIIINP species that is higher in molecular weight than Coll but smaller than intact PIIINP. The exact nature of the antigen and the basis of this interaction are not further explained. The recognition of a discrete intermediate PIIINP species further underlines the heterogeneity of the circulating PIIINP antigens and emphasizes the need to clearly identify the nature of the epitope that is recognized in each case.

From the available literature it becomes clear that monoclonal antibodies with well defined binding epitopes that recognize human PIIINP have not been reported so far.

The lack of detailed binding information sheds doubts on the diagnostic value of PIIINP determinations using these antibodies.

State of the art: monitoring fibrotic disease processes

A diverse array of diseases is associated with the inappropriate or unregulated production of collagen. PIIINP measurements can be performed from patient sera or other body fluids from patients with these diseases. Among these are liver fibrosis of various etiologies, alcoholic cirrhosis, biliary cirrhosis, hepatitis, schistosomiasis, cardiac fibrosis of various etiologies, idiopathic interstitial fibrosis, idiopathic pulmonary fibrosis, interstitial pulmonary fibrosis, acute pulmonary fibrosis, acute respiratory distress syndrome, perimuscular fibrosis, pericentral fibrosis, dermato-fibroma, kidney fibrosis, diabetic nephropathy, glomerulonephritis, systemic and localized scleroderma, keloids, hypertrophic scars, severe joint adhesions, arthrosis, myelofibrosis, corneal scaring, cystic fibrosis, muscular fibrosis, Duchenne's muscular dystrophy, esophageal stricture, retroabdominal scaring, Crohn's disease, ulcerative colitis, aneurysms of large vessels.

Further fibrotic disorders can be induced or initiated by scar revisions, plastic surgeries, glaucoma, cataract fibrosis, corneal scaring, joint adhesions, graft vs. host disease, tendon surgery, nerve entrapment, Dupuytren's contracture, OB/GYN adhesions, pelvic adhesions, peridural fibrosis, diseases of the thyroid gland or the parathyroids, metastatic bone disease, multiple myeloma, and restenosis.

Early diagnosis is essential for a potential treatment of these diseases. Up to the present, serum tests for fibrotic diseases are not well established and final diagnosis must entirely rely on invasive biopsies.

PIIINP measurements can also be performed to measure the rate of collagen synthesis in patients undergoing therapy with glucocorticosteroids.

Furthermore, the antibodies directed against PIIINP can be used to assess collagen synthesis in tissue samples from patients with fibrotic disease by immunohistochemical staining of kryostat and paraffine sections.

Immunoassay applications:

The immunoassay of the invention comprises reaction of two antibodies with a human fluid sample, wherein the capture antibody specifically binds to the 30 most N-terminal amino acids of the PIIINP molecule. This capture antibody preferentially binds to trimeric PIIINP. A second antibody of different epitope specificity is used to detect this complex. Preferably the antibodies are monoclonal antibodies and both of said two antibodies of the assay specifically bind to the protein.

The antibody or antibodies of the assay that specifically bind to the PIIINP preferably exhibit less than about 3% cross-reactivity, in an assay such as described in example 6 below or a similar assay with a human plasma sample, with PIIICP, PICP, collagen (III), collagen (I) and collagen (VI).

"Antibody", "antibody of the invention" or other similar term as used herein includes a whole immunoglobulin as well as antigenic binding fragments or immunoreactive fragments which specifically bind to the PIIINP, including Fab, Fab', $F(ab')_2$ and F(v).

The human fluid sample used in the assay of the invention can be any sample that contains the PIIINP, e.g. blood or urine. Typically a serum or plasma sample is employed.

Antibodies of the invention can be prepared by techniques generally known in the art, and are typically generated to a sample of PIIINP. The antibodies can also be generated from an immunogenic peptide that comprises one or more epitopes of the PIIINP that are exhibited by native PIIINP.

More particularly, antibodies can be prepared by immunizing a mammal with a purified sample of PIIINP, or an immunogenic peptide as discussed above, alone or complexed with a capture. Suitable mammals include typical laboratory animals such as sheep, goats, rabbits, guinea pigs, rats and mice. Rats and mice, especially mice, are prefered for obtaining monoclonal antibodies. The antigen can be administered to the mammal by any of a number of suitable routes such as subcutaneous, intraperitoneal, intravenous, intramuscular or intracutaneous injection.

Preferably immunization is done by subcutaneous, intraperitoneal, or intravenous injection. The optimal immunizing interval, immunizing dose, etc. can vary within relatively wide ranges and can be determined empirically based on this disclosure. Typical procedures involve injection of the antigen several times over a number of weeks. Antibodies are collected from serum of the immunized animal by standard techniques and screened to find antibodies specific for PIIINP. Monoclonal antibodies can be produced in cells which produce antibodies and those cells used to generate monoclonal antibodies by using standard fusion techniques for forming hybridoma cells. Typically this involves fusing an antibody-producing cell with an immortal cell line, such as a myeloma cell, to produce the hybrid cell. Alternatively, monoclonal antibodies can be produced from cells by the method of Huse *et al.* (1989).

One suitable protocol provides for intraperitoneal immunization of a mouse with a composition comprising of purified PIIINP conducted over a period of about two to seven months. Spleen cells then can be removed from the immunized mouse. Sera from the immunized mouse are assayed for titers of antibodies specific for PIIINP prior to excision of spleen cells. The excised mouse spleen cells are then fused to an appropriate homogenic or heterogenic (preferably homogenic) lymphoid cell line having a marker such as hypoxanthine-guanine phosphoribosyltransferase deficiency (HGPRT) or thymidine kinase deficiency (TK). Preferably a myeloma cell is employed as the lymphoid cell line. Myeloma cells and spleen cells are mixed together, e.g. at a ratio of about 1 to 4 myeloma cells to spleen cells. The cells can be fused by the polyethylene glycol (PEG) method. The thus cloned hybridoma is grown in a culture medium, e.g. RPMI-1640 (Moore *et al.*, 1967).

Hybridomas, grown after the fusion procedure, are screened, e.g. by radioimmunoassay or enzyme immunoassay, for secretion of antibodies that bind specifically to PIIINP, e.g. antibodies are selected that bind to the complete PIIINP, to region I of the subsequence but not to region II. Preferably an ELISA is employed for the screen. Hybridomas that show positive results upon such screening can be expanded and cloned by limiting dilution method. Further screens are preferably performed to select antibodies that can bind to PIIINP in solution as well as in human fluid samples.

The assay of the invention is illustrated by the following protocol using alkaline phosphatase as the label of the conjugate antibody. A test sample, e.g. a human serum sample, is added to a PIIINP antibody (i.e., an antibody that binds to the PIIINP) bound on a capture such as a microtiter plate and the antibody-antigen reaction is conducted, followed by addition of the alkaline phosphatase-labeled PIIINP antibody conjugate obtained as outlined above, and then a further antibody-antigen reaction is performed. The antibodies are typically dissolved in solution prior to contact with a test sample. Suitable diluents include those known in the art for use in immunoassays. A specifically prefered solution for dissolving the antibodies for contact with a test sample contains 20 mM Tris, 500 mM sodium chloride, 0.05 mg/ml mouse IgG and 5% (w/v) BSA.

Preferably both the capture and conjugate antibodies of the assay of the invention specifically bind to the PIIINP, and the antibody bound to the support, the human fluid sample and labeled antibody are incubated together, followed by wash steps to remove any unbound labeled antibody and the human plasma sample other than the reacted PIIINP. Suitable washing agents include those known in the art for use in immunoassays. A specifically prefered washing buffer solution contains 27.2 g/l imidazole, 17.5 g/l sodium chloride and 4 ml/l Tween 20. If necessary, a substrate for alkaline phosphatase is added to the assay and the reaction products are assayed for enzyme activity by measuring the absorbance or fluorescence of the resulting product. The detected amount of bound labeled antibody is directly proportional to the concentration of PIIINP in the assayed human serum sample. Thus a quantitative deter-

mination of the PIIINP concentration in the plasma test sample can be determined by comparison of the absorbance or fluorescence of the test sample with absorbance values obtained from standardized solutions that contain known amounts of PIIINP. It may be desirable to prepare calibration curves from absorbance values obtained from a number of standardized solutions to facilitate interpretation of values obtained from a test sample.

A specifically prefered immunoassay of the invention was conducted as follows. The capture monoclonal antibody and conjugate monoclonal antibody labeled with alkaline phosphatase (AP) was incubated together with a human plasma sample at 37°C for 30 minutes. The plate was then washed and incubated for 15 minutes at room temperature with AP substrate and the bound conjugate was quantitated.

PIIINP can be purified from a human serum sample by use of capture complexes coupled with one or more antibodies of the invention in sufficient quantities for immunization. Sufficient amounts were purified and hence the invention includes methods for obtaining purified PIIINP using the antibodies of the invention and related apparatus. A suitable purification procedure provides coupling an antibody of the invention on an appropriate capture as is known in the art, such as a gel or resin, then packing the capture in a column, and then eluting a sample solution containing PIIINP through the column to selectively adsorb PIIINP. The antibody can be suitably coupled onto the capture by known methods, e.g. the cyanogen bromide method, glutaraldehyde method, aqueous carbodiimide method, active ester method, and the like. The antibody also may be physically adsorbed on the surface of the capture.

Example 1. Production of Recombinant PIIINP

Cloning of PIIINP cDNA

PCR was carried out following the manufacturer's recommendations for buffer conditions with 2.5 units of *Taq* polymerase (Boehringer Mannheim, Germany). Two specific primers with *Kpn*I and *Hin*dIII restriction sites integrated at their respective 5' ends (P1 and P5, table 1) were added to a final concentration of 500 fM each. 1 µl of QUICK-Clone cDNA from human aorta was used as a template in a 100 µl PCR reaction. PCR was carried out with a high performance UNO thermocycler manufactured by Biometra, Germany. The following program was used: initial template denaturation step : 5 min at 94°C. Standard cycle conditions were: 45 sec at 94°C, 60 sec at 55°C, 60 sec at 72°C. 30 Cycles were carried out. A 420 sec extension step completed the program. The resulting PCR fragment was purified from a 2% agarose gel with a QIAEX II gel extraction kit (Qiagen, Germany) and phosphorylated by T4 polynucleotide kinase treatment (Boehringer Mannheim, Germany). The acterial phagemid vector pBluescript SK$^-$ was digested with the resriction enzyme *Sma*I (Boehringer Mannheim, Germany), and the linearized plasmid was dephosphorylated with calf intestine phosphatase from Boehringer Mannheim, Germany. The enzymatically modified PCR fragment and the vector were ligated with T4 ligase (Boehringer Mannheim, Germany) for 12 h at 16°C. All enzymatic modification steps were carried out according to the manufacturer's recommendations. The ligated plasmid was transformed into the *E. coli* strain sure II (Stratagene, USA) according to the procedure described in Sambrook *et al.*, 1988. The resulting colonies were expanded in LB medium with 100 µg/ml Ampicillin and the plasmid DNA was isolated according to the Qiagen Mini Kit protocol (Qiagen, Germany). The plasmid was tested for the integration of the insert by digestion with *Kpn*I and *Hin*dIII (Boehringer Mannheim, Germany) according to the manufacturer's recommendations. A positve clone (*hP5*) was identified. The clone was expanded, sequence-verified by two-directional nucleotide sequencing in an Applied Biosystems (USA) sequencer, and larger quantities of the *hP5* plasmid were isolated. It served as a template for all expression constructs described below.

Subsequently, the plasmid *hP5* served as a template for the generation of an N-terminal His tag expression construct encompassing the entire propeptide region but lacking the N-terminally located presequence and the telopeptide region distal to the N-proteinase cleavage site. To this end, 1 µg of the *hP5* plasmid was used as the template and the amplification was carried out with the primers P3 and P14 (see table 1 for sequence information). P3 and P14 contained *Kpn*I and *Hin*dIII restriction sites at their ends, respectively. 12 PCR cycles were carried out under the same cycle conditions as described above. This PCR product was blunt-endedly subcloned into the phagemid vector pBluescript SK⁻. A plasmid containing the cDNA of mature PIIINP was obtained and designated 4.5. This plasmid was digested with *Kpn*I and *Hin*dIII (Boehringer Mannheim, Germany) and the PIIINP cDNA was purified from a 2% agarose gel. The insert was subsequently phosphorylated. It was ligated with the His tag expression plasmid pQE30 (Qiagen, Germany) that had previously been digested with the same set of restriction enzymes as the insert, and dephosphorylated. Competent *E. coli* sure II were transformed with the plasmid. A colony carrying the pQE30 derivative with the PIIINP sequence was identified and expanded. All modification steps were carried out in anology to the procedure outlined for the generation of *hP5*. The clone was designated *4.5.2*.

Expression and purification of the recombinant N-terminal His tag PIIINP fusion protein and generation of N- and C-terminally truncated mutants

Briefly, after a second transformation, the corresponding N-terminal His tag fusion protein was expressed in the *E. coli* strain M15 carrying the pREP4 plasmid (according to the QIAexpressionist manual, p. 35, Qiagen, Germany, incubation temperature 37°C). The recombinant fusion protein was purified over a Ni-NTA Superflow column (Qiagen, Germany) according to the purification protocol from the manufacturer (Protocol 7, QIAexpressionist manual, pp. 45-46, Qiagen, Germany).

For the generation of an N-terminally truncated protein, a primer recognizing a sequence downstream of the sequence coding for the first 30 N-terminal amino acids

of PIIINP (P11-2, table 1) was used in combination with a primer specific for the sequence encompassing the C-terminally located N-proteinase cleavage site (P14, table 1). The experimental procedures were completely analogous to the those described for the construction of the plasmid *4.5.2* except that the insert was subcloned into the pQE31 expression vector (Qiagen, Germany) and that the upstream primer (P11-2) contained a *Pst*I restriction site at its 5' end. Therefore, the insert and the vector were digested with *Pst*I (Boehringer Mannheim, Germany) rather than *Kpn*I. This construct was designated *clone6*. After induction with IPTG an N-terminal fusion protein lacking the first 30 amino acids from the Col1 domain of PIIINP and differing from the *4.5.2*-derived peptide in the amino acid sequence adjacent to the N-terminal His tag amino acid sequence was purified over a Ni-NTA column.

Another truncated PIIINP cDNA sequence was generated by PCR using a primer pair recognizing the sequence coding for the N-terminal end of the molecule (P3, table 1) and the sequence just upstream of the sequence coding for the entire Col2 domain (P12, table 1). After subcloning this fragment into the pQE30 expression vector, transformation, expression of the recombinant protein and its purification, a truncated PIIINP lacking 21 amino acids at its C-terminus when compared to the non-truncated molecule was obtained. The construct was designated *2.8.6*. Again, all experimental procedures were carried out in analogy to the ones described for the preparation of the *4.5.2* plasmid.

Multimerization attempts with rhPIIINP

The purified proteins were separated by electrophoresis on a 12.5% SDS gel (Sambrook *et al.*, 1989). To determine which molecular weight disulfide-linked PIIINP species and truncated mutants were recombinantly expressed in the strain M15 with the pREP4 plasmid, mercaptoethanol [5% (v/v)] as the reducing agent was omitted from the sample buffers and the resulting bands were compared with a molecular size standard and with their mercaptoethanol-reduced counterparts.

By comparison of the unreduced PIIINP species with the reduced protein, that appeared as one band on an SDS gel, it became apparent that the recombinant PIIINP was largely synthesized as a monomeric protein. Only species with electrophoretic mobilities corresponding to monomers were discernable in each case (figure 3).

Amino acid sequencing of the purified recombinant proteins

Sample preparation of PIIINP to remove buffer and other contaminants was performed using the ProSpin sample preparation cartridge from Applied Biosystems (USA) and Centricon concentrators (Amicon, USA), by blotting onto PVDF-membrane after SDS-gel electrophoresis or by gel filtration in formic acid on a silica gel column. A vertical electrophoresis unit (LKB/Pharmacia, Germany), a trans blot cell and a power supply for blotting model 250/2.5 (BioRad, Germany) were used. The reagents for electrophoresis and the PVDF-membrane were from BioRad. All other chemicals used were of pro analysis or biochemical grade (Merck, Germany). The prestained protein marker was from BioRad (Germany).

N-terminal sequence analyses of PIIINP-fragments were performed using the gas-liquid-solid-phase protein sequencer 473A from Applied Biosystems. The standard sequencer program was used. The sequencer, the different running programs, the cycles, as well as the PTH-separation system, are described in detail in the respective manual (User's manual protein sequencing system model 473A (1989), Applied Biosystems, USA). The detection of PTH-amino acids was performed on-line using an RP-18-column (220 mm x 2 mm, 5 µ-material) PTH-column from Applied Biosystems. The PTH-amino acids were identified and quantified by a 50 pM standard of all PTH-amino acids. The data were collected and integrated using the sequencer data system 610A from Applied Biosystems. About 20 ng of the respective PIIINP fragments was used for sequencing.

The *4.5.2*, *2.8.6*, and *clone6* proteins were N-terminally sequenced as described above. The number of cycles was sufficient in each case to reach the collagen

sequence of the fusion protein. Table 2 summarizes the results from the amino acid sequencing of three representative PIIINP proteins. It was identical to the published sequence in each case.

Example 2: Immunization

BALB/c female mice were immunized with PIIINP to provide spleen donors for the PEG fusion, disclosed below, that generated monoclonal antibodies that specifically bind to PIIINP.

BALB/c female mice were sensitized with 10 µg each of purified PIIINP in immunogen emulsion prepared as follows:
0.125 ml procollagen-III-N-terminal-propeptide (325 µg/ml in 50 mM Tris/50 mM NaCl/0.1 mM EDTA pH 7.4)
1.500 ml Complete Freund's Adjuvant
1.375 ml Dulbecco's Phosphate Buffered Saline
Each mouse was injected with 0.5 ml of this immunogen emulsion i.p. The mice were boosted with doses of up to 50 µg of purified PIIINP i.p. in an immunization protocol that was carried out for approximately six months.

Example 3: ELISA Assay for PIIINP Antibodies

Coating Microtiter plates with PIIINP

PIIINP was diluted in a coating buffer (carbonate buffer, pH9) at a concentration of 1 µg/ml. 100 µl of each solution (10 ng PIIINP) were placed in each well of the microtiter plates, which were sealed and incubated overnight at 2-8°C. The contents of the wells were then aspirated and the plates were washed once with wash/storage buffer, the wash aspirated, and the plates again resealed. The plates were stored at 2-8°C until use.

Example 4: Preparation of Hybridomas

Hybridomas secreting monoclonal antibodies to procollagen-III-N-terminal-propeptide were generated by two cell fusions. The PEG fusion technique was employed. The myeloma cells used were HRPT-minus P3 - X63-Ag8.653 (P3X) (ATCC CRL1580). Selection for hybrids was accomplished using HAT media (hypoxanthine, aminopterin and thymidine). Unfused P3X myeloma cells will not survive in this medium as they lack the apparatus to use hypoxanthine to produce purines. The aminopterin present in the medium blocks the endogenous synthesis of purines and pyrimidines.

Fusion to form hybridoma procollagen-III-N-terminal-propeptide

Splenocyte preparation

Spleen cells were obtained from a mouse immunized with procollagen-III-N-terminal-propeptide as described for Example 1. The cells were released from the spleen using a forceps and needle, then suspended in 12 ml of cold 20% Complete Medium without serum (RPMI 1640 base, Gibco). The cells were then centrifuged at 200g for 10 minutes after which the supernatant was removed by aspiration, and the cells were resuspended again in cold medium. This washing process was repeated twice, and the cells resuspended in a final volume of 10 ml. The viable cell count of the splenocytes was 1.7×10^8 at a viability of 98% by Trypan Blue exclusion technique.

Myeloma preparation

The myeloma cells were harvested mechanically, pooled, and centrifuged at 200g for 10 minutes after which the supernatant was removed by aspiration, and the cells were resuspended in 50 ml of 20% Complete Medium. The viable cell count of the myeloma cells was 2.9×10^6 viable cells per ml at a viability of 76%.

Fusion of the splenocytes and myeloma cells

The splenocytes and 15 ml of the myeloma cell suspension were combined at a spleen cell to myeloma cell ratio of approximately 4:1 with a total viable cell count of

2.13×10^8. The volume was brought up to 50 ml with cold 20% Complete Medium without serum and the cells then centrifuged at 200g for 10 minutes. The cell pellet was then washed twice with this medium at a volume of 50 ml. After the final wash, the supernatant was removed by aspiration and the pellet centrifuged at 200g for 3 minutes and the remaining supernatant aspirated. The cells were then fused with 40% PEG (molecular weight 7,000 to 9,000) buffered in RPMI 1640; fusion was performed in a tube held in a warm (37°C) water bath. 1 ml of PEG solution warmed to 37°C was added to the pellet and incubated for 1 minute. The PEG solution was then diluted by addition of 20 ml of warm 20% Complete Medium without serum. The fused cells were incubated at 37°C for 10 minutes and then centrifuged at 200g for 10 minutes.

The fused cell pellet was then resuspended in 50 ml of 20% Complete Medium and plated at cell densities of 1.09×10^5 to 4.26×10^5 per well. The cells were plated in a final volume of 200 µl per well of 20% Complete Medium with 250 units/ml IL-6. After overnight incubation at 37°C and 10% CO_2, one half of the medium in each well was aspirated and the cells were fed with 20% HAT, 20% HAT with 5 µg/ml STM or 20% HAT with 500 units/ml IL-6. The cells were visually scanned and fed periodically with these media for several weeks, while the growth of the hybridomas was monitored and growing wells were screened for the presence of anti-PIIINP antibodies beginning at day 12 to 14 post-fusion using the previously described PIIINP-coated microtiter plates and standard methods well known to the biochemical experts.

Example 5: Application of Recombinant PIIINP and of the Truncated PIIINP Peptides for the Characterization of the Epitope-Specificity of Monoclonal Antibodies Raised Against Recombinant PIIINP

Principle

To screen for epitope-specific monoclonal antibodies, all three recombinant peptides were used. Those monoclonal antibodies recognizing all three peptides were not used any further because these antibodies were either directed against the N-terminal His

tag or against the region between the very N-terminal epitopes of Col1 and the beginning of Col2. Antibodies recognizing only the complete peptide (*4.5.2*) and the C-terminal deletion peptide (*2.8.6*) lacking the 21 most C-terminal amino acids corresponding to a deletion of the entire Col2 domain and not reacting with the N-terminal deletion peptide (*clone6*) lacking the 30 most N-terminally located amino acids were classified as epitope-specific for the N-terminal Col1 region. Figure 2 shows the epitopes present on all PIIINP constructs and table 4 summarizes the results from ELISA and Western blot assays obtained with the different antibodies and antigens.

By the same token, antibodies recognizing only the complete peptide (*4.5.2*) and the N-terminal deletion peptide (*clone6*) were identified as epitope-specific for the C-terminally located Col2 domain.

To characterize the binding characteristics of the newly generated monoclonal antibodies, both ELISA and Western Blot techniques were applied. Two suitable monoclonal antibodies (mAb 35J22 and mAb 35J23) that exclusively recognized the 30 most N-terminally amino acids of PIIINP as their binding epitopes and that allowed the sensitive detection of native PIIINP from patient samples were selected:

1. ELISA:

After identification of the optimal titer using complete recombinant PIIINP in an ELISA assay where the recombinant PIIINP was coated at a fixed mass concentration, the deletion peptides were coated at the same concentration, and the signal intensity was determined in exact analogy to the complete PIIINP. Table 4 summarizes the results of this ELISA assay for the monoclonal antibody 35J23 and recognition of the different antigens.

2. Western Blot:

After determination of the optimal titers of the different antibodies all three PIIINP variants were electrophorized in equal molar concentrations and Western Blots were performed. Differences in antigen recognition could thus be correlated with epitope-specificity.

Example 6: Immunization with an Oligopeptide to Raise Antibodies that are Selective for the Col2 Domain

In order to raise antibodies selective for the Col2 domain an oligopeptide sequence corresponding to the 21 amino acids located immediately N-terminal to the N-proteinase cleavage site was chosen. The exact sequence of the peptide is given in table 3. The last 14 amino acids of the oligopeptide sequence are identical to the sequence of the Col2 domain of PIIINP. As the binding epitope of a typical monoclonal antibody is at least 6 amino acids, antibodies raised against this oligopeptide typically recognize a subsequence of the Col2 region.

A monoclonal antibody, mAb 35JC2 was raised and further characterized.

Example 7: Establishment of a PIIINP Immunoassay Based on the Recognition of Two Complementary Epitopes of the PIIINP Molecule by Two Different Monoclonal Antibodies

Measurements of the N-terminal procollagen (III) propeptide (PIIINP) concentrations in patients' sera were carried out employing the sandwich technology. An epitope-specific monoclonal antibody recognizing an N-terminal epitope of the PIIINP molecule (mAb 35J23) specifically bound and immobilized the circulating serum PIIINP. A second monoclonal antibody (mAb JC2) was used to detect this complex.

The combination of the site-specific monoclonal antibodies as described above exclusively recognized higher molecular weight species of PIIINP that are related to

the *de novo* deposition of procollagen (III) in the extracellular matrix in the sera of all patients and controls. This specificity for intact PIIINP further distinguishes the novel assay from assays that additionally recognize lower molecular weight species. Shorter PIIINP fragments probably represent degradation products emanating from PIIINP and may not necessarily reflect recent collagen synthesis. Therefore, the new assay described above is very specific for the purpose of monitoring collagen synthesis versus collagen breakdown.

Example 8: Establishment of a PIIINP Immunoassay Based on the Capture of the PIIINP Molecule by a Monoclonal Antibody and Detection of this Complex with Polyclonal Antiserum

A microtiter plate (Nunc Maxisorb, Germany) was coated with mAb 35J23 or mAb 35J22 (2µg/ml, total volume per well 100 µl) overnight at 4°C. On the following day the supernatant was discarded and the wells were blocked with 3% BSA (w/v) in PBS (total volume per well 200 µl) for 2 h. The wells were washed 3x with washing buffer (0.05% Tween 20, BioRad, Germany). Subsequently, 50 µl of patient serum was applied to each well for 1 h. The serum was removed from the wells and the plates were washed 3x with washing buffer as described above. The polyclonal anti-PIIINP antiserum from Biodesign (UK, lot # 40331R) was used to detect this complex (dilution 1:500 (v/v), total volume per well 100 µl). After 3x washing, the peroxidase-labeled anti-rabbit antibody A 0545 from Sigma (Germany) was applied (dilution 1:20,000 (v/v), total volume per well 100 µl). After 5x washing the ELISA assay was developed with 100 µl of TMB peroxidase substrate + peroxidase solution B (1:1 (v/v), both from Kirkegaard & Perry Laboratories, USA) for 30 min. The reaction was stopped by the addition of 100 µl of 1 N H_2SO_4 and the O.D.'s were determined at 450 nm.

Most surprisingly, both monoclonal antibodies, mAb 35J22 and and mAb 35J23, preferentially bound to trimeric PIIINP vs. the monomeric recombinant protein 4.5.2.

Example 9: PIIINP Assay on an Automated Immunoanalyzer

Figure 3 and table 4 show the correlation of the O.D.'s obtained with this assay with the PIIINP concentrations determined with the Orion RIA when samples from the same selected patients were measured with both methods.

The calibration curve shown in figure 4 was obtained with recombinant PIIINP when tested with the ELISA assay described in Example 8. The assay was performed as described above for patient sera, except that successive dilutions of recombinant PIIINP were coated (total volume 100 µl). The titer of the polyclonal anti-PIIINP antiserum was varied in this experiment. Best results were obtained with 1:500 dilutions of the anti-PIIINP antiserum. When the results are compared to the measurements from patient sera with known PIIINP concentrations it becomes clear that this type of assay preferentially recognizes intact PIIINP while not avidly recognizing monomeric recombinant material. With the recombinant antigen the detection threshold was approximately 100 ng/ml and the dynamic range of the assay was shifted to PIIINP concentrations 20-100 times higher than with the trimeric material in patient sera. Notably, the preferential recognition of the trimeric material is not due to the polyclonal antiserum that does not discriminate between monomeric and trimeric material as has been shown in simple ELISA assays performed with only the polyclonal antiserum (data not shown).

Table 5 shows the PIIINP concentrations in the sera from patients with Child-classified fibrotic liver disease. The data imply a correlation between circulating PIIINP levels as determined with the sandwich immuno assay described above and the clinical severity of the fibrotic liver disease.

The PIIINP assay was set up as a sandwich immunoassay with simultaneous addition of both antibody reagents and sample, and late addition of magnetic particles.

- 10 µl serum sample and 10 µl of magnetic particle buffer were pipetted into the reaction cuvette.

- 30 sec. later 65 µl reagent R1 and 65 µl reagent R2 were dispensed into the cuvette. Reagent R1 contains a fluoresceinated monoclonal anti-PIIINP antibody in a buffered solution. Reagent R2 contains an anti-PIIINP antibody of different epitope-specificity conjugated to alkaline phosphatase in a buffered solution.

- The reaction mixture was incubated for 21 min at 37°C to form the sandwich immune complex.

- 10µl of magnetic particles coated with an anti-fluorescein antibody were added, and the mixture was incubated at 37°C for additional 8 min.

- The immune complex bound to the particles was separated from the reaction mixture by applying an external magnetic field. The particles were washed to remove excess sample and reagent.

- 300 µl p-nitrophenolate was added to the reaction mixture. The colored p-nitrophenolate anion was formed, and the rate of formation was directly proportional to the PIIINP concentration present in the sample. At low concentrations the rate of absorbance increase was monitored at 405 nm, at high color formation rate the filter wavelength was switched to 450 nm.

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Tables

Table 1

Primer Sequences

hP5 (template extending from secretion sequence to telopeptide region)
P1 and P5

P1: 5'- CGCG GGT ACC AAG GGG AGC TGG CTA CTT CTC -3'
P5: 5'- CGCG AAG CTT AGG ATA GCC TGC GAG TCC TCC -3'

4.5.2 (entire cDNA sequence)
P3 and P14

P3: 5'- CGCG GGT ACC CAG GAA GCT GTT GAA GGA GGA -3'
P14: 5'- CGCG AAG CTT GGG AGA ATA GTT CTG AGG AC -3'

Clone6 (N-terminal deletion of 30 aa C-terminally adjacent to secretion leader sequence)
PP11-2 and P14

P11-2: 5'- CGCG CTG CAG TGT GAC TCA GGA TCC GTT CT-3'
P14: 5'- CGCG AAG CTT GGG AGA ATA GTT CTG AGG AC -3'

2.8.6 (C-terminal deletion of 21 aa N-terminally adjacent to N-proteinase cleavage site)
P3 and P12

P3: 5'- CGCG GGT ACC CAG GAA GCT GTT GAA GGA GGA -3'
P12: 5'- CGCG AAG CTT AGG GGA CCC TGG TTG TCC T -3'

Table 2

PIIINP *4.5.2* Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser-Ala-Cys-Glu-Leu-Gly-Thr-**Gln-Glu-Ala-Val-Glu-Gly-Gly-...**

PIIINP *2.8.6* Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser-(Ala)-(Cys)-Glu-Leu-Gly-Thr-**Gln-Glu-Ala-(Val)-Glu-Gly-...**

PIIINP *clone6* Met-Arg-Gly-Ser-His-His-His-His-His-His-Thr-Asp-Pro-His-Ala-Ser-Ser-Val-Pro-Arg-Val-Asp-Leu-Gln-...

Table 2 shows the results of the N-terminal sequencing of the PIIINP His tag fusion proteins. The amino acids from the collagen sequence are printed in **bold letters**. Amino acids that were not directly discernable in the procedure but whose presence can be infered from the subsequent sequencing cycle results are printed in brackets.

Table 3

Antigen	Antibodies mAb 35J23 and mAb 35J22 directed against N-terminal epitope [Reactivity]	Antibody 35JC2 directed against C-terminal epitope [Reactivity]
4.5.2 (intact PIIINP with 6xHis tag)	+++	+++
PIIICP4.1 (unrelated antigen with identical His tag sequence)	-	-
2.8.6 (21 aa C-terminal deletion protein)	+++	-
clone6 (30 aa N-terminal deletion protein)	-	+++

Table 3 summarizes the results from ELISA assays measuring the reactivity of the two monoclonal antibodies against recombinant *4.5.2* and against the deletion proteins. The N-terminal amino acid sequences were identical in all cases up to the beginning of the collagen sequence. The control protein, PIIICP4.1, was an N-terminal 6xHis tag fusion protein identical to the PIIINP proteins in its N-terminal non-collagen-sequence and was used to ascertain that the antibodies were not directed against the His tag sequence.

Table 4

O.D. (sandwich ELISA)	PIIINP concentration according to Orion assay [ng/ml]	calculated PIIINP concentration based on sandwich ELISA calibrated by regression curve [ng/ml]
0.543	2.6	-1.9
0.794	5.3	5.5
0.761	7.0	4.5
1.210	11.1	17.7
1.328	16.7	22.0
1.190	21.9	17.1
1.692	29.6	31.8
1.773	36.8	34.2

Table 4 shows the results of PIIINP serum concentration measurements performed with the commercially available Orion assay and with the sandwich ELISA using the monoclonal antibody mAb 35J23 and polyclonal anti-PIIINP serum. The assays are in excellent agreement (r=0.94). The third column depicts the calculated PIIINP concentration as estimated with the sandwich ELISA assay.

Patient Child classification	calculated PIIINP concentration based on sandwich ELISA calibrated by regression curve [ng/ml]
A	below detection threshold
A	2.5
B	below detection threshold
B	38.4
B	47.5
C	below detection threshold
C	28.4
C	35.7
C	42.1
C	44.2

Table 5 shows the results of PIIINP serum concentration measurements in sera from patients with fibrotic liver diseases. The patients were classified according to the clinical Child classification. Measurements were performed with the sandwich ELISA with the monoclonal antibody mAb 35J23 and polyclonal anti-PIIINP serum (Biodesign, U.K.). The assay was calibrated with sera where the PIIINP concentration was determined with the Orion RIA kit and the sandwich ELISA using the mAb 35J23 and polyclonal anti-PIIINP antiserum. The correlation between O.D.'s in the ELISA assay and the values from the Orion kit was excellent ($r=0.76$, data not shown here).

Figures

Figure 1 shows the N-terminal cDNA sequence of preprocollagen (III) extending beyond the telomer region. The different primer locations described above are indicated by arrows pointing in the direction of polynucleotide chain extension.

Figure 2 schematically shows the cDNA regions encoded by each of the constructs *hP5*, *4.5.2*, *2.8.6*, and *clone6*.

Figure 3 shows the results of PIIINP serum concentration measurements performed with the commercially available Orion assay and their correlation with the results from the sandwich ELISA with the monoclonal antibody mAb 35J23 and polyclonal anti-PIIINP antiserum. The assays are in excellent agreement (r=0.94).

Figure 4 shows the relationship between the absorptions measured with the sandwich ELISA assay with the monoclonal antibody mAb 35J23 in combination with polyclonal anti-PIIINP serum and the coating concentrations of recombinant monomeric PIIINP (*4.5.2*).